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Genetics

Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I

(homologous recombination/gene targeting/lipoproteins)

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ABSTRACT **Atherosclerosis is a major cause of morbidity and mortality in developed countries. In humans the risk of atherosclerosis is inversely correlated with plasma levels of high density lipoprotein (HDL). As a step in determining whether the experimental reduction of plasma HDL level will increase susceptibility to atherosclerosis, we have used gene targeting in embryonic stem cells to produce mice lacking apolipoprotein A-I, the major protein component of HDL particles. Mice homozygous for the disrupted gene have no plasma apolipoprotein A-I detectable by double immunodiffusion; their total plasma cholesterol and HDL-cholesterol levels after overnight fasting are reduced to about one-third and one-fifth of normal levels, and they are grossly deficient in α-migrating HDL particles.**

Inactivation of a specific gene in the genome of mouse embryonic stem (ES) cells by homologous recombination (gene targeting) followed by the generation of animals carrying the mutated gene is providing an increasing store of biological insights (1, 2). The relevant technology has assisted the understanding of the developmental roles played by genes, such as oncogenes (3–5) and homeobox genes (6), and knowledge about the immunological importance of molecules, such as β_2-microglobulin (7, 8) and interleukin-4 (9), has been greatly enhanced. Animals carrying a specific single-gene defect in an otherwise normal genetic background are, therefore, expected to be useful for studying the role of the defect in the etiology of human genetic disease. Relationships between genotypes and phenotypes are generally direct when the disease is caused by a single-gene defect, as is exemplified by the hemoglobinopathies. However, when more than one gene is involved and particularly when environmental factors play an important role, the relationships between genotypes and phenotypes become much more complex. Examples include diabetes, hypertension, and atherosclerosis. Gene-targeting experiments can, nevertheless, provide another approach to unraveling the complex etiology of such multifactorial diseases. Individual candidate genes can be modified, and their isolated effects in the living animal can be determined in controlled environments. Even more powerfully, single-gene modifications can be combined by breeding, so that eventually the genetics of the multifactorial disease can be deciphered.

The present experiment, generation of mice carrying an inactivated apolipoprotein A-I gene (*Apoa-1*), is a step toward dissecting the role of genetic factors involved in lipid metabolism and atherogenesis. Apolipoprotein A-I (apoA-I), synthesized in the liver and small intestine, is the major protein complexed with high density lipoprotein (HDL) in mammals (10). In addition to its role in lipoprotein metabolism as a structural protein, apoA-I also participates in cholesterol ester formation by serving as a cofactor for lecithin–cholesterol acyltransferase (11). In humans an inverse correlation between the risk of developing atherosclerosis and plasma levels of HDL has been observed (12). Human individuals deficient in apoA-I caused by several different types of mutation in the gene appear to be predisposed to atherosclerosis (13–18). These observations point to the importance of apoA-I and HDL particles in atherogenesis.

The negative correlation between HDL levels and atherosclerosis observed in humans is supported by differences in susceptibility to dietary-induced atherosclerosis in mice of different strains. Thus C57BL/6 mice are more susceptible than are C3H or BALB/c mice (19), and this susceptibility segregates with a genetic determinant, *Ath-1*, that appears to control the plasma level of HDL in response to high fat diet (20). Furthermore, C57BL/6 mice show a 2-fold lower HDL-cholesterol level after being fed an atherogenic diet than do mice of the other two strains. Finally, evidence that apoA-I expression is a major determinant of atherosclerosis comes from the protective effects against high dietary lipids afforded by over-production of human apoA-I in transgenic mice (21); this leads to as much as 3-fold higher levels of total and HDL cholesterol (21, 22). As a first step to examine the effects of absence of apoA-I on atherogenesis in mice, we have constructed mice having their *Apoa-1* gene inactivated by gene targeting. Here we report these experiments, which have produced mice lacking in plasma apoA-I and grossly deficient in their plasma HDL levels.

MATERIALS AND METHODS

Cloning of the Mouse *Apoa-1* Locus and Construction of the Targeting Plasmid. A 9-kilobase (kb) *Bam*HI fragment of mouse genomic DNA containing the *Apoa-1* gene and a part of the gene for apolipoprotein C-III was isolated by screening a λ phage library made from DNA of a strain 129/Ola mouse. We determined the nucleotide sequence of ≈5 kb of the fragment to establish its validity; the sequence is ≈89% identical to the equivalent region of the rat sequence (23). A targeting construct designed to disrupt the endogenous *Apoa-1* locus after the homologous recombination was made by replacing the 400-base-pair (bp) *Eco*RV–*Eco*RI fragment containing exon 2 of the gene with the positively selectable neomycin resistance gene (pMC1neopolyA, Stratagene). A herpes simplex virus thymidine kinase (TK) gene was placed either at the 5′ or 3′ end of the construct to make 5′TK or 3′TK constructs, respectively.

Gene Targeting and Screening of the Homologous Recombinants. ES cell (E14TG2a) culture and electroporations were

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Abbreviations: apoA-I, apolipoprotein A-I; ES cells, embryonic stem cells; HDL, high density lipoproteins; TK, thymidine kinase.

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done as described (24, 25). Before electroporation, the targeting plasmids were linearized at a *Not* I site in the vector. Ten to twelve days after electroporation, colonies resistant to G418 (200 μg/ml, Sigma) and to 2 μM ganciclovir (from Syntex, Palo Alto, CA) were picked and passaged in clonal fashion. DNA was isolated for Southern analysis from 60-mm dishes of nearly confluent ES cells by standard methods or from 20-mm dishes by a salting-out method (26). Three micrograms of DNA was digested with restriction enzymes, and Southern blots were made using conventional procedures.

Generation of Germ-Line-Competent Chimeras. Approximately 10 ES cells were injected into the blastocoele cavity of C57BL/6J embryos. Surviving blastocysts were transferred to the uteri of pseudopregnant CD-1 or C57BL × CBA F_1 females. An average of two to three transfers were made per cell line. Animals chimeric by coat color were bred to C57BL/6J animals to determine their germ-line competency.

Measuring Total and HDL Cholesterol. Total plasma cholesterol levels were measured enzymatically by using 1 or 2 μl of plasma and 100 μl of commercially available reagents (Sigma). HDL-cholesterol levels were measured after removing apolipoprotein B-containing particles by precipitation with polyethylene glycol (21). Measurements were made in duplicate and averaged for samples collected from 8-week-old homozygotes and heterozygotes derived from two F_1 heterozygote matings. Values for seven normal animals of approximately the same age but from other $F_1 \times F_1$ matings are given for comparison. Before fasting overnight, the animals had been on breeding chow (ProLab 2000 formula), which contains ≈9% crude fat.

RESULTS

Targeting constructs were made by using a 9-kb *Bam*HI fragment isolated from strain 129 mouse genomic DNA that contains the complete *Apoa-1* gene plus 5'- and 3'-flanking region sequences (including part of the gene for apolipoprotein C-III). Exon 2 of the *Apoa-1* gene in the fragment was replaced by sequences from pMC1neopolA (27) that confer resistance to G418 in mammalian cells. These sequences are in the same transcriptional orientation as the *Apoa-1* gene. A herpes simplex virus TK gene was placed either at the 5'TK or at the 3'TK of the construct to allow use of a positive/negative-selection strategy with G418 and ganciclovir (28). Fig. 1 illustrates the scheme of homologous recombination by using the 5'TK construct and shows the positions of probes used in analysis of the experimental data.

The targeting DNA was introduced into ES cells (E14TG2a) (29) by electroporation, and DNA samples from ES cell colonies resistant to G418 and ganciclovir were assayed for targeting by Southern blotting (Fig. 2). Probe 1 hybridizes to a single 12.0-kb *Hin*dIII fragment in DNA from parental cells (lane 6 of Fig. 2*a*) but hybridizes to this fragment and to an additional 4.6-kb fragment of equal intensity in DNA from targeted cells (lanes 2–5 in Fig. 2*a*). DNA from cells that have incorporated the targeting DNA elsewhere in the genome, by a nonhomologous event, give *Hin*dIII fragments of unpredictable sizes, as exemplified by lane 1 in Fig. 2*a*. Targeting was confirmed by the presence of an 8.5-kb *Hin*dIII fragment after hybridization to probe 2, probe 3, or to a probe specific for the *neo* gene (data not shown), and by Southern blots of digests with other restriction enzymes. For example, Fig. 2*b* shows additional *Nhe* I and *Bcl* I fragments of the expected sizes in DNA from two of the targeted cells in addition to fragments of the same size as those from parental cells; hybridization was to probe 1.

Table 1 summarizes the targeting data from five experiments, the first two with the 5'TK construct and the remaining three with the 3'TK construct. As the data show, the frequency of targeting the mouse *Apoa-1* locus was very high, ranging from 82% (experiment 1) of colonies that survived double selection when using the 5'TK construct to 46%

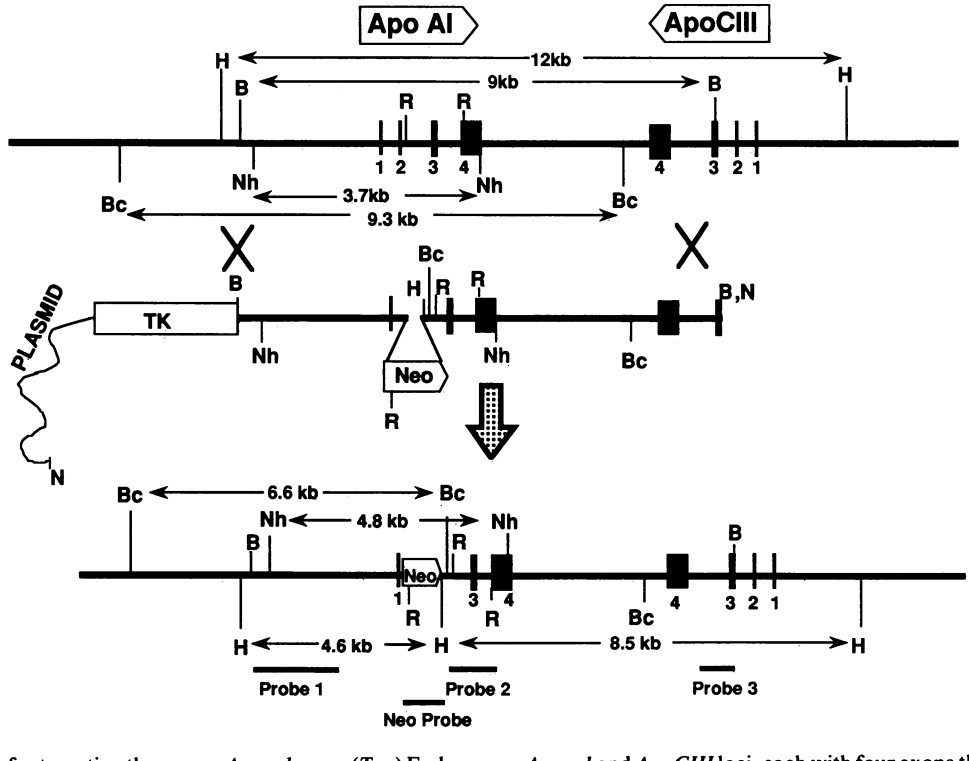

FIG. 1. Strategy for targeting the mouse *Apoa-1* gene. (*Top*) Endogenous *Apoa-1* and *ApoCIII* loci, each with four exons that are convergently transcribed. (*Middle*) Targeting construct. The construct contains a *Hin*dIII and a *Bcl* I site at the 3' end of the *neo* gene that are not present in the native locus. (*Bottom*) A correctly targeted locus depicted after a homologous crossover. Restriction fragment lengths of parental and modified loci used to identify the correct modification are indicated. Probes 1, 2, and 3 are a 2-kb *Bam*HI–*Xba* I fragment, a 1-kb *Eco*RI fragment, and a 600-bp *Kpn* I–*Bam*HI fragment, respectively. B, *Bam*HI; Bc, *Bcl* I; H, *Hin*dIII; N, *Not* I; Nh, *Nhe* I; and R, *Eco*RI.

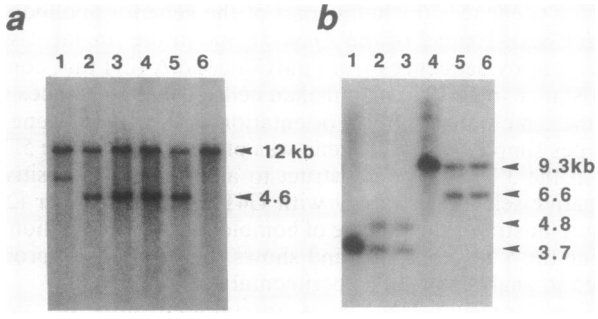

FIG. 2. Southern blot analysis of DNA from ES cell colonies. (*a*) Southern blot of genomic DNA from six ES cell colonies digested with *Hin*dIII and hybridized to probe 1 (Fig. 1). Parental cell DNA is in lane 6; the 12-kb band is from the unmodified *Apoa-1* gene. Lanes 2–5 contain DNA from colonies that have been correctly targeted; a 4.6-kb band is present in addition to the 12-kb band. Lane 1 is DNA from a colony in which the targeting DNA was incorporated into the genome somewhere else, giving a band of unpredictable size, while leaving the 12-kb parental band intact. (*b*) The planned modification was confirmed by Southern blot analysis of DNA digested with other restriction enzymes. The blot was hybridized with probe 1. Lane 1 is DNA from parental cells digested with *Nhe* I; lanes 2 and 3 are DNA from targeted colonies digested with *Nhe* I; lane 4 is DNA from parental cells digested with *Bcl* I; lanes 5 and 6 are DNA from targeted colonies digested with *Bcl* I.

(experiment 5) with the 3′TK construct. Homologous recombination occurred under our conditions approximately once per 10^5 treated cells. Ganciclovir selection gave a 2- to 4-fold enrichment over that seen with G418 alone, as demonstrated in experiment 3, in which 19% (8/42) of G418-resistant cells were targeted.

When injected into blastocysts, the targeted cell lines varied in the extent of chimerism produced in resulting offsprings. Two out of nine lines used for injection were germ-line competent: one of them gave three male chimeras able to transmit the ES cell genome to their offspring; another gave one germ-line-competent female chimera. Southern blot analysis of DNA isolated from the tails of ES cell-derived offspring of a male chimera mated to C57BL/6J females showed that ≈50% of them inherited the inactivated *Apoa-1* allele.

Animals homozygous for the modified *Apoa-1* gene were generated from matings of heterozygotes. Southern blot analysis of DNA isolated from the tails of nine pups from two litters is shown in Fig. 3*a*. In four of these samples (pup numbers 27–29 and 32), probe 3 (see Fig. 1) hybridized to a single 8.5-kb *Hin*dIII fragment; this result establishes that these animals are homozygous for the modified *Apoa-1* gene. Pups 30, 31, 34, and 35 were heterozygotes (8.5-kb and 12-kb bands), and number 33 had no modified genes (12-kb band only). Animals homozygous for the disrupted *Apoa-1* appear healthy at their present age (2–3 mo).

Mice homozygous for the disrupted *Apoa-1* gene have no apoA-I in their plasma detectable by Ouchterlony double-immunodiffusion tests against rabbit anti-mouse apoA-I antiserum (Fig. 3*b*), although strong precipitation is clearly visible with plasma from animals heterozygous or homozygous for the normal gene. This result establishes that at the protein level the planned modification eliminated apoA-I production, as was expected from the design of the targeting construct.

At the lipid level, we find (Table 2) that the total cholesterol in plasma from homozygous mutants at 8 weeks of age is ≈33% that of normal animals of similar age ($P < 0.0005$). HDL-cholesterol levels in the animals show an even greater reduction (17%, $P < 0.0005$). Heterozygous animals have total cholesterol and HDL-cholesterol levels 54% and 40%, respectively, of the levels in normal animals.

Electrophoresis of plasma samples on nondenaturing polyacrylamide gradient gels (Fig. 3*c*) shows that the homozygous animals (animals 27–29 and 32) are grossly deficient in HDL particles. There is also an overall reduction of the HDL particles in heterozygotes (animals 30, 31, and 34) in comparison with normal (animal 33), but the size distribution of particles in heterozygotes is not markedly altered.

DISCUSSION

We have used gene targeting to produce mice lacking apoA-I. The overall efficiency of targeting the *Apoa-1* gene in mouse ES cells was one in 10^5 of the treated cells. About one-fifth of ES cell colonies surviving a single positive selection and about one-half of surviving double-positive-negative selection were correctly targeted. We used isogenic DNA as a source of targeting DNA and used ≈2.5 kb and 6 kb of genomic sequences at each side of the *neo* gene to facilitate the homologous recombination. Targeting frequencies similar to those we report here have been demonstrated for the *pim-1* protooncogene (30) but can be orders of magnitude less. With the same ES cells, similar lengths of sequence homology, and essentially identical experimental conditions, the frequencies of obtaining targeted cells has varied, in our own and our colleagues' hands, from one in 10^5 treated cells for the *Apoa-1* gene (this paper), one in 10^6 for the hypoxanthine phosphoribosyltransferase gene (31) and the apolipoprotein E (*Apoe*) gene (24) and less than one in 10^7 in the cystic fibrosis transmembrane regulator gene (B. Koller, personal communication). All the targeting constructs used in these experiments were isogenic, except for that with the *Apoe* gene. It is not understood at this point what makes one locus more accessible to homologous recombination than another; it may be a property of the locus and/or of the sequence in the incoming DNA. Experiments dissecting the *Apoa-1* gene region with a view to identifying elements that influence the frequency of homologous recombination events would be worthwhile.

Animals homozygous for the inactivated *Apoa-1* gene appear healthy at age 3 mo, although they lack apoA-I protein in their plasma and have only one-fifth of HDL cholesterol compared with normal. Heterozygotes also have their HDL-cholesterol levels reduced (to 40%). That the mutant animals appear healthy is very important. This fact proves that apoA-I is not vital to the normal development of mice, confirming the deduction one would make from the existence

Table 1. Homologous recombination of the mouse *Apoa-1* gene

Exp.	DNA (conc.)	Cells treated	Selection	Colonies surviving, no.	Colonies analyzed, no.	Targeted colonies, no. (%)
1	5′TK (5 nM)	4×10^7	G418 + Ganciclovir	150	28	24 (82)
2	5′TK (2 nM)	3×10^7	G418 + Ganciclovir	2500	50	35 (70)
3	3′TK (2 nM)	2.5×10^7	G418	1253	42	8 (19)
4	3′TK (2 nM)	5.8×10^7	G418 + Ganciclovir	1240	47	29 (62)
5	3′TK (2 nM)	2.9×10^7	G418 + Ganciclovir	851	48	20 (46)

Exp., experiment; conc., concentration.

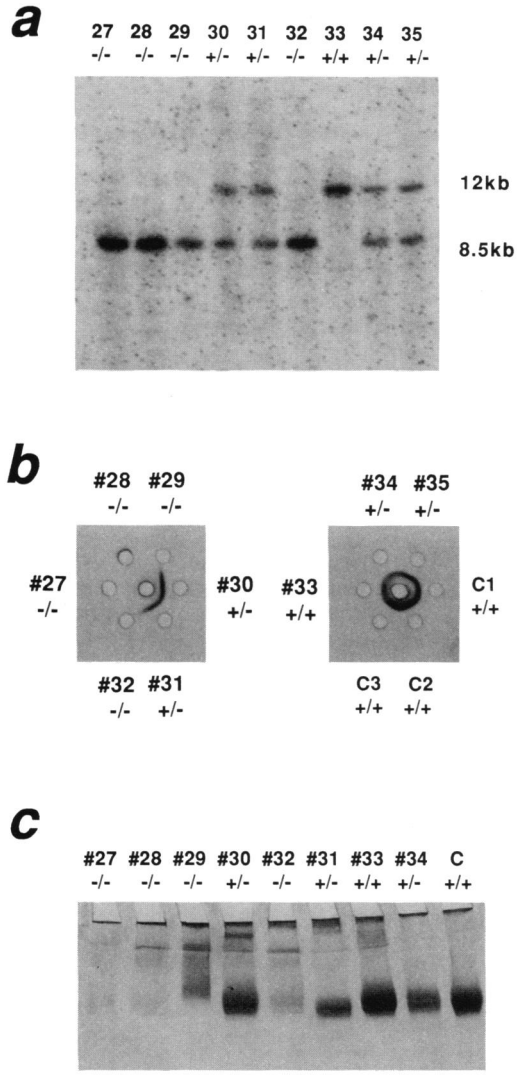

FIG. 3. (*a*) Southern blot analysis of tail DNA. DNA samples were isolated from the tails of 3-week-old animals resulting from two heterozygote matings. Probe 3 (Fig. 1) was used to identify the 8.5-kb *Hin*dIII band diagnostic of the modified *Apoa-1* gene. Animals 27–29 and 32 are homozygous for the mutation (−/−); animals 30, 31, 34, and 35 are heterozygous (+/−); animal 33 is homozygous normal (+/+). (*b*) Ouchterlony double-immunodiffusion precipitation of plasma from nine pups (animals 27–35) from heterozygote F_1 matings and from three control mice (C1–C3). Plasma (0.2 μl) was diluted with phosphate-buffered saline and placed in the peripheral wells made in 1.0% agarose/phosphate-buffered saline/3% polyethylene glycol (PEG 8000) (24); 5 μl of antiserum was in the central well. −/−, +/−, and +/+ indicate samples, respectively, from homozygous mutants, heterozygous mutants, and nonmutant animals. (*c*) Native gel electrophoresis of plasma. Plasma samples (15 μl) were collected after overnight fasting from the same animals at 8 weeks of age as were used for the Ouchterlony test and were prestained with Sudan black. Electrophoresis was done in a 4–20% gradient polyacrylamide gel (Bio-Rad) by using Tris/glycine buffer at pH 8.3.

of humans who lack apoA-I. Survival of apoA-I-deficient animals will enable detailed studies of the consequences in mice of apoA-I deficiencies in lipid metabolism and atherogenesis. In humans lacking apoA-I, a marked reduction of HDL cholesterol has been observed. Changes in the total cholesterol in these individuals (≈67%; ref. 32) are, however, not so obvious as in our mutant mice (33% in homozygotes and 54% in heterozygotes). This difference may be a consequence of the fact that the major cholesterol carrier in mice is HDL, whereas in humans it is low density lipoprotein (ref. 33). However, the two species are similar in that the lack of apoA-I does not appear to affect the amount of lipoprotein particles other than HDL. Humans lacking apoA-I suffer from premature coronary heart diseases. It will, therefore, be of great interest to determine whether the homozygous or even heterozygous mutants having reduced levels of plasma HDL develop atherosclerosis spontaneously as they age, as well as to investigate how they respond to atherogenic diets.

Table 2. Cholesterol levels in plasma of animals carrying mutant *Apoa-1* genes

Genotype (*n*)	Total cholesterol, mean mg/dl ± SD	HDL cholesterol, mean mg/dl ± SD
Homozygote (4)	28.7 ± 8.6 ($P < 0.0005$)*	13.2 ± 4.6 ($P < 0.0005$)
Heterozygote (3)	47.5 ± 9.2 ($P < 0.05$)	30.3 ± 13.5 ($P < 0.01$)
Control (7)	88.3 ± 18.0	75.6 ± 16.2

*P values are against controls.

No strains of mice are known that develop atherosclerosis spontaneously when fed standard chow (≈5% in fat). A high-fat diet (≈15%) induces fat accumulations in the aorta of some strains of mice that are equivalent to early lesions seen in human atherosclerosis. We anticipate that the inactivated *Apoa-1* gene will have different effects in different genetic backgrounds.

It will be of interest to backcross the apoA-I-deficient F_1 heterozygotes to normal C57BL/6J animals so that the effects of the mutation can be studied in the C57BL/6J background, which is associated with susceptibility to dietary-induced atherosclerosis. It will also be important to study the effects of the mutation in the moderately resistant 129 background by mating chimeras and their offspring solely to strain 129 animals. Furthermore, studying mice carrying the null *Apoa-1* allele together with a null apolipoprotein E (*Apoe*) allele (24) should be of great interest. Apolipoprotein E-deficient mice are expected to have elevated levels of plasma cholesterol as a result of a reduced ability to clear chylomicron remnants and very low density lipoprotein remnants. The combination of these two defects, both potentially atherogenic, should, therefore, increase the likelihood that animals will be obtained that develop atherosclerosis spontaneously, even when fed a normal diet.

In conclusion, we have used homologous recombination in ES cells to generate mice carrying a disrupted *Apoa-1* gene. These mice should be of great use in dissecting the role of apoA-I in lipid metabolism. Study of the effects of our inactivated *Apoa-1* gene mutation in different normal genetic backgrounds and in combination with other potentially atherogenic mutations should allow better understanding of the complex process of atherogenesis. Such animals should be valuable models for the development of additional drugs and therapies for reducing the incidence of coronary heart disease.

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